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14. ABSTRACT
Our objective is to create a multi-institutional tissue microarray resource from radical prostatectomy samples with detailed clinical information and follow-up and rigorous case-cohort design for use as a platform for validating tissue biomarkers of prognosis. In addition, we have proposed testing a series of biomarkers of prognosis and a set of biomarkers that correlate with Gleason Score. We have made significant progress over the past year. Having completed construction of the tissue microarrays and finalized standard procedures for tissue microarray storage, sectioning and shipping, we have now stained scanned, gridded and read TMAs for several biomarkers. We havenow changed to the Leica scanner and PathXL image analysis software suite for some of the biomarkers and have also used the Aperio system for others. Pathologists have read compete sets of TMAs for H & E, High Molecular Weight Keratin, ERG, SPKINK1, Ki67 (MIB1), Survivin and PTEN FISH and we have correlated staining results with clinical outcome. We also have made significant progress in testing TACOMA, an automated TMA scoring algorithm. We have completed staining of the TMAs for . Over the next year we will complete refinements of the infrastructure, complete pathologic review of the p27 and MUC1 biomarkers, and stain and evaluate several additional biomarkers which have received approval. We will complete statistical analysis for all of the completed biomarkers (and others evaluated over the next year) and plan to publish papers for each of the biomarkers over the next year. We wil also carry out outcome analysis for a panel of the biomarkers soon.

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Validation of Biomarkers for Prostate Cancer Prognosis

Synergy Award: W81XWH-11-1-0380

Co-PIs: James D. Brooks & Ziding Feng

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Introduction

As discussed in our progress report last year, the debate surrounding PSA testing has intensified by the final D rating by the US Preventative Services Task Force – stating that PSA testing should not be done because the risks of testing outweigh the benefits. In large part, these recommendations are based on entrenched practice patterns in which nearly all men diagnosed with prostate cancer are treated based on the uncertainty regarding the long-term clinical outcome of men with low and intermediate risk prostate cancer. Standard treatments, mainly surgery and radiation therapy, result in well documented significant morbidities, including significant lower urinary tract symptoms such as incontinence and urinary urgency as well as sexual dysfunction. Furthermore, evidence from many sources suggests that most prostate cancers are relatively indolent, and men will often succumb to other causes of death. However, PSA screening continues to be widely practiced and patients and physicians view the test as better than nothing. Therefore, PSA testing is likely to continue despite USPSTF recommendations. One possible solution to the screening problem is to increase the use of active surveillance (AS) in men with low and very low risk cancers. Acceptance of AS can be enhanced by tests of prognosis that provide some index of risk of the cancer. Recently, Myriad Genetics (Prolaris) and Genomic Health (OncotypeDx Prostate) have introduced gene expression tests that can be performed on biopsies and provide a score of risk. These tools have limited data validating their use in selectiung AS. Furthermore, they are very expensive, sometimes cannot be run on small amounts of tissue, and require shipment and processing of biopsies. It is widely recognized that imunohistochemical markers would provide a less expensive assessment of prognosis that could be run on-site and can be run on small amounts of cancer tissue. Unfortunately, at this point, there are few validated immunohistochemical markers of prognosis, although many have been proposed.

To address this challenge, we began our multi-institutional Canary Tissue Microarray Project. We have used rigorous clinical trial case/cohort design, taking care to correct for institutional and spectrum biases. Funding from the Department of Defense allowed us to complete construction of the TMAs as well as the necessary infrastructure and begin testing biomarker candidates. With this infrastructure in place, we now have a robust validation platform for testing prostate cancer biomarkers. We hope and intend that this resource will be a source for future biomarker validation studies even after the DOD funding has ceased. We are pleased to report our progress after 2 years.

Specific Aim 1) To test markers of prognosis on prostate cancer tissue microarrays with associated clinical data.

1.A. Develop work-flow for TMA sharing, image scanning, TMA staining data analysis.

The multi-institutional TMAs have been constructed at all sites. The final TMA cohort is 1326 patients, of which 1232 have clinical data. We are in the process of updating follow-up on the TMAs since several years of additional follow-up have been

accumulated since the cases were first selected. Patients have been selected at random from the pool of patients who had undergone radical prostatectomy at each of the sites, with special attention to selecting patients with features typical of low-intermediate risk patients seen in contemporary urologic practices. Details of patient selection, statistical considerations, and TMA construction are summarized in our publication in Advances in Anatomic Pathology published earlier this year and appended to last year's report. In addition to this cohort, a separate TMA has been constructed from 220 patients who underwent radical prostatectomy at a sister site who have very long term follow-up (up to 25 years) and hard endpoints including metastases and prostate cancer specific death. Since many of these patients were diagnosed in the pre-and early PSA eras, they are held separately as a validation cohort.

We have completed several stated aims in the proposal with regard to development of work-flow for array sharing, analysis and archiving while some aspects continue to be developed:

1) After TMA manufacture was completed, Standard Operating Procedures (SOPs) for TMA storage, sectioning and transferal have all been working well at each site. Staining for the biomarkers currently under evaluation has been universally good, as detailed below.

2) Slide shipping works well for sending slides to investigators for staining, as well as to the image scanning centers.

3) We have completed H & E staining of the complete set at Stanford University. In addition, we have stained the complete set for high molecular weight keratins (HMWK) to aid the pathologists in interpreting slides.

4) Image capture and archiving has been completed. As detailed in last year's report, we have changed to the Leica SCN400 Slide Scanner with the SL801 Autoloader based at UBC. The Leica scanner can capture high resolution images of an entire TMA in about 1 hour and is fully automated so a deck of slides can be loaded and scanned. The images generated are automatically ported into the PathXL image analysis software suite (http://www.pathxl.com/index.php/pathxl-research/pathxl-tma). This system has the advantage of flexibility in setting up scoring parameters and image manipulation that STMAD lacked. In addition, this system incorporates abilities to directly correlate clinical data with staining data.

Now that this system is in use, we continue to improve the interface for the pathologists. One big challenge was that there was no easy way to display the H & E, HMWK and biomarker cores simultaneously. Pathologists frequently need to refer to the H & E and HMWK stains in order to judge the presence and location of cancer in the TMA core. This information is needed in scoring each core for the marker of interest. To solve this problem, we have had the pathologists go through the H & E and HMWK stains and score each core for the presence and location of the cancer. We are currently changing the PathXL screen so that the results of these reads are available for each core. With

these results available, the pathologists will no longer need to refer to the H & E and HMWK stains in order to score a core for a particular analyte. This will save considerable time for the pathologists.

5) One major challenge has been the considerable time required of the pathologists to simply read the TMAs. As mentioned above our TMAs have 1326 patients represented, each with 4 cores. In other words: **1326 pts (x 4 cores) = 5304 cores**. This is a considerable number of cores for the pathologists to read. If they also include H & E and HMWK the work becomes overwhelming, i.e.: **1326 pts (x 4 cores)= 5304 cores (x 3 stains) = 15912 stains**. We are attempting to overcome this with porting into the database the HMWK and H & E staining results so that the pathologists no longer need to look at these while scoring. Regardless, the reading of 5304 cores requires a single pathologist on average approximately 70 hours to look at and score all of the cores. This time commitment is significant, especially considering that the pathologists are not being paid from this or any grant to perform the reads. This has proven to be a major bottleneck in working through candidate biomarkers – yet we have had growing success.

Furthermore, we are attempting to overcome this formidable task of reading TMAs by adding an automated commercial system for reading TMAs that is from Aperio. This scanner allows quantification of colors in a core and can be used for quantitative reads of staining intensity. In addition, the system allows identification of nuclei so that percentage of positive nuclei, in addition to staining intensity, can be collected and quantitated. We have used this system for Ki67 (MIB1) staining and are about to adapt it for p27 staining. See below and Dr. Feng's report for discussion of these results.

6) We have looked at inter-observer variability in reading IHC stains for ERG. In this experiment, we had 7 pathologists score one TMA (200 cores) for ERG staining, a biomarker with highly robust and reproducible staining. In the first round, pathologists scored the TMAs according to their own systems – without prior discussion of the methods they would use for assessing positive, intermediate and negative cores. The agreement was good, but modest. In a second round, pathologists agreed upon scoring metrics and the concordance increased significantly, with near complete agreement between the pathologists.

7) Data management: The clinical data are complete for the TMAs and have been used by Dr. Feng for analysis of staining results of the TMAs, as detailed in his report. One change is that Dr. Feng has moved from Fred Hutchinson Cancer Research Institute to MD Anderson Cancer Center. He will discuss the transfer of the DMCC to MDACC.

8) TACOMA progress will be reviewed by Dr. Feng in his report.

1.B. Test candidate biomarkers of prognosis for prediction of recurrence after radical prostatectomy

In monthly conference calls, the TMA investigators review progress and review applications for utilizing the TMAP resource. Most applications for use of the TMAs

come from within the group, although it is available to the prostate cancer research community broadly and can be accessed by application through the Canary Foundation website (http://www.canaryfoundation.org). We have focused on biomarkers that have well characterized, highly performing reagents (e.g. immunohistochemical grade antibodies) and sufficient preliminary data that they could supply prognostic information independent of grade, stage and PSA. We have begun staining for biomarkers listed in our proposal.

1) Proposed biomarkers: We have completed immunohistochemical staining for ERG, SPINK1, p27 (KIP1), MUC1 and Ki67 (MIB1). In all cases, the staining was at exceptionally high quality per initial review of the glass slides by our pathologists. Scores will be correlated with clinical outcome. Since our TMA is uniquely designed for high level validation of markers, we intend to publish finding whether positive or negative so that poorly performing biomarkers can be discarded. In addition to immunohistochemistry, Dr. Jeremy Squire at Queens University, Ontario, Canada has completed FISH to interrogate copy number alterations (allelic loss) at the PTEN locus. The pathologists have completed reads of the slides for PTEN FISH, Ki67 (MIB1), and ERG. These data and their correlation with clinical outcomes are reviewed in Dr. Feng's progress report. We anticipate that each of these biomarkers will result in a high impact publication that we anticipate submitting over the next several months. We are well under way for completing analysis for P27 (KIP1) and SPINK and anticipate these will also be published. Furthermore, we plan to perform an integrated analysis of all biomarkers to generate a model of prognosis.

2) We have approved applications for several biomarkers and will carry these out over the next year. These include ARG2 and CD38, CD10, SMAD7, and 2 different approaches to analysis of stromal components (Trichrome stain and image analysis software that works from H & E slides). We are also going to perform an analysis of AZGP1 by both RISH and using IHC. AZGP1 has been shown to be prognostic in several datasets and was originally described by the Brooks group in 2004. It is part of the Genomic health gene panel in OncotypeDx. Several other applications are currently being evaluated.

Specific Aim 2) To evaluate candidate markers that correlate with Gleason grade on prostate cancer tissue microarrays with associated clinical data.

Thus far, we have focused on building the analysis pipeline and in staining high priority biomarkers of prognosis. The intent of this aim is to investigate biomarkers that correlate with Gleason grade. Several markers are in our queue and are listed in the original proposal. For some, we are still looking for high quality affinity reagents that provide interpretable staining with limited background. Leading candidates are AGR2, a marker expressed at high levels in Gleason pattern 3 cancers and Monoamine oxidase A, expressed at high levels in Gleason pattern 4 disease.

For all biomarkers, whether for Gleason score or prognosis, the statistical analysis strategy has been outlined in our proposal and will be used as soon as reads are available from the pathologists.

Key Research Accomplishments

- Completion of construction of TMAs at all participating sites
- Standardizing and deploying Standard Operating Procedures for TMA storage, sectioning and shipping at each site
- Centralized shipping, collation and distribution of TMAs at Stanford University
- Biomarker review and approval by the investigative team to ensure quality of the reagents and sufficient level of evidence for investigation of a particular biomarker on our valuable resource.
- Inclusion of investigators in the broad prostate cancer research community for testing candidate biomarkers. Groups using the resource include Dr. Jeremy Squire, Dr. Gustavo Ayala, and Dr. Lidong Liu.
- Porting final clinical data that will be used for analysis of biomarker performance to the MD Anderson DMCC.
- Deployment of a more efficient image capture system (Leica) so that we can increase the throughput of biomarker testing.
- Use of the Aperio image analysis system with Ki67 (MIB1) with plans to adapt to p27 (KIP1)
- Customization and use of a new image archiving and displaying software for management and scoring of the immunohistochemical staining by the study pathologists
- Completion of foundational staining for H & E and HMWK. Ina ddition, we have completed the pathologist interpretation of the cores for each of these stains and are incorporating these in the database to be made available for the patholosts to use in interpreting each core on the TMA for new stains.
- Completion of staining for ERG, SPINK1, p27 (KIP1) and Ki67 (MIB1) MUC1. We anticipate publishing separate manuscripts for ERG and SPINK, p27, and Ki67.
- Completion of TMA analysis by the pathologists for PTEN FISH, ERG, Ki67.
- Preliminary correlation of staining and clinical data for the above biomarkers.

Reportable Outcomes

1) Publications referencing this grant:

James D. Brooks: Translational genomics: The challenge of developing cancer diagnostic biomarkers. *Genome Research* **22:** 183-187, 2012.

Sarah Hawley, Ladan Fazli, Jesse K. McKenney, Jeff Simko, Dean Troyer, Marlo Nicolas, Lisa F. Newcomb, Janet E. Cowan, Luis Crouch, Michelle Ferrari, Javier Hernandez, Antonio Hurtado-Coll, Kyle Kuchinsky, Janet Liew, Rosario Mendez-Meza, Elizabeth Smith, Imelda Tenggarra, Xiaotun Zhang, Peter R. Carroll, June M. Chan, Martin Gleave, Raymond Lance, Daniel W. Lin, Peter S. Nelson, Ian M. Thompson, Ziding Feng, Lawrence D. True and James D. Brooks: Design and construction of a resource for the validation of candidate prognostic biomarkers: the Canary Prostate Cancer Tissue Microarray as a model. *Advances in Anatomic Pathology* **20:** 39-44, 2013.

James D. Brooks: Managing localized prostate cancer in the era of prostate specific antigen testing. *Cancer*, In press, 2013.

Conclusion

We have undertaken a challenging task of creating a multi-institutional TMA resource with rigorous case/cohort design. To our knowledge, such a resource has not been previously created and offers the advantage of reducing institutional biases as well as spectrum biases. In the uniform design and through image acquisition and archiving technologies, we have created a resource that can be easily used by the greater prostate cancer research community. In many ways, this resource represents a gold standard by for evaluation of prognostic biomarkers. We have completed all phases of pipeline construction and continue to refine our work-flow to improve functionality as we work with the resource. We now have tested several biomarkers and confirmed that they are prognostic. We will complete analysis of the biomarkers in the context of the clinical data over the next year and plan several publications. In addition, we will continue to carry out analysis of new biomarkers and solicit applications for biomarkers inside and outside our research group. This research directly addresses the PCRP overarching challenge to *distinguish lethal from indolent disease*.